The Genetic Identification of Species of Agronomic *Azolla* Lam. Indigenous to Mexico

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The aquatic fern *Azolla* Lam. is familiar in tropical agriculture as a nitrogen biofertilizer in rice paddies of Southeast Asia (IRRI, 1987). Its symbiosis with the nitrogen-fixing cyanobacterium *Anabaena azollae* Strasb. exploits a renewable resource. Expansion of *Azolla* useage to other rice-growing regions is a realistic possibility when this fern is also utilized as animal or poultry feed, or in a tripartite rice-fish-*Azolla* system.

Agricultural application of *Azolla* is considered feasible in the neotropics. In Mexico, native *Azolla* species were found to grow rapidly, fix atmospheric nitrogen in significant amounts, and increase rice yield (Ferrera-Cerrato, 1980; Quintero Lizaola & Ferrera-Cerrato, 1987; Quintero Lizaola et al., 1990).

The difficulty of species identification, however, hinders the selection of desireable *Azolla* for field use. The morphological plasticity of New World taxa limits the utility of sporophytic characters for identification. Nonetheless, *A. filiculoides* (FI), *A. caroliniana* (CA), and *A. mexicana* (ME) have in the past been reported to be indigenous to Mexico (Svenson, 1944; Sota, 1976). Using molecular criteria, CA, ME, and *A. microphylla* (MI) are now known to be very genetically similar. They may be regarded as a "species complex", while FI is genetically distinct (Zimmerman et al., 1991a, b). The range of MI, when treated as a separate species, is believed to exclude Mexico (Schofield and Colinvaux, 1969).

This paper classifies and fingerprints *Azolla* collected in Mexico for agricultural purposes, using a diagnostic system of molecular markers. Growth results demonstrate the potential for this biofertilizer in rice agriculture of Mexico.

METHODS

Collection of Mexican Azolla. — The original 13 accessions of Mexican *Azolla* (Table 1) were deposited with the Centro de Edafologia del Colegio de Postgraduados (CEDAF). Most of this germplasm came from central and southern Mexico; *Azolla* from Sinaloa was the exception (Fig. 1). Some characterization was completed on newer accessions as they became available (Table 1). A number of these *Azolla* were obtained from the International Rice Research Institute (IRRI), which also possesses duplicates of various CEDAF accessions. Eight *Azolla* from IRRI had been tentatively identified as FI, CA, ME, or MI from morphological features.

Maintenance and Growth. — Fern sporophytes were maintained as clonal cultures at CEDAF under greenhouse conditions, and indoors at the University of Michigan-Dearborn. The latter environment included a mixture of natural sunlight and cool-white fluorescent lamps (12 h:12 h cycle) and a temperature of 25°C. Isolates were grown in N-free rice culture growth medium (Yoshida et al., 1971) in Mexico and N-free BG-11 medium (Rippka et al., 1979) in the USA.

Relative growth rates (RGR) of *Azolla* were estimated as changes in dry weights from

Table 1. Locale, coordinates, and altitude of each original *Azolla* collection site (01-13), and origins of newer accessions.

Key	Locale of collection	Latitude north			Longitude west			Altitude (m)
01 - CH	Chignahuapan, Puebla	19°	50'	16"	98°	00'	57"	2320
		19°	50'	44"	98°	01'	24"	
02 - JA	Cerro Gordo Mpio. de Emiliano Zapata, Veracruz.	19°	21'	50"	97°'	14'	20"	1435
03 - MO1	Atlacomulco, Mpio. de Jiutepec, Morelos	18°	46'	28"	99°	14'	47"	1350
04 - OA	Sta. Cruz. Mixtepeo, Mpio. de Zimatlán, Oaxaca	16°	48'	50"	96°	54'	12"	1500
05-SI1	Guamúchil, Sinaloa.	25°	26'	00"	108°	04'	32"	50
		25°	27'	13"	108°	05'	16"	
06 - TA1	Mazatiupa region, H.	17°	59'	30"	92°	26'	54"	10
	Cárdenas, Tabasco.	18°	05'	30"	92°	48'	35"	
07 - HI1	17 Km northwest of Ciudad	19°	53'	20"	98°	21'	54"	2520
	Sahagún in Laguna de	19°	50'	08"	98°	25'	44"	
	Tecocomulco, Hidalgo.							
08 - TE	Km 60, highway from	19°	17'	30"	98°	25'	45"	2300
	Mexico-Puebla, Texmelucan,							
	Tlaxcala	19°	18'	05"	98°	26'	30"	
09 - TL	Tláhuac, Distrito Federal.	19°	15'	25"	99°	02'	38"	2240
10 - TO	Sn. José Toshi, Edo. De	19°	51'	44"	99°	56'	20"	2550
	Mexico.	19°	52'	05"	99°	56'	38"	
11 - VI	Km 26.5, federal highway	19°	09'	50"	96°	15'	45"	18
	Veracruz-Xalapa, Mpio. de	19°	09'	58"	96°	15'	59"	
	Manlio Favio Altamirano,							
	Veracruz.							
12 - V2	Mpio. de Manlio Favio,	19°	21'	55"	97°	16'	10"	18
	Altamirano, Veracruz.							
13 - XO	Xochimilco, Distrito Federal.	19°	15'	10"	99°	05'	10"	2240
14 - MO2	Atlacomulco, Morelos	Site Date Unknown						
15 - TA2	Ceicades, H. Cardenas,	"						
	Tabasco							
16 - MO3	Atlacomulco, Mpio. de	"						
	Jiutepec, Morelos							
17 - MO4	Ticuman, Morelos	"						
1510 - HI2	Hidalgo	"						
2501 MC	Mexico City, Distrito Federal	"						
3510 TA3	Tabasco	"						
3519, 3520	Unspecified Collection Sites	"						
4506 - SI12	Sinaloa	"						
8001 - V3	Veracruz							
8031 - V4	Veracruz	"						

greenhouse cultures (Evans, 1972; Wong Fong Sang et al., 1987). Nitrogen accumulation was determined by RGR and %N (Bremner, 1965).

Fingerprinting

DNA polymorphisms. — Molecular evaluations were first conducted on the original 13 CEDAF accessions, which at the time composed all of the available Mexican *Azolla*. The procedures for harvesting and purifying DNA from *Azolla* have been described, as have the protocols for the analysis of DNA restriction fragment length polymorphisms (RFLPs) (Zimmerman et al., 1991b).

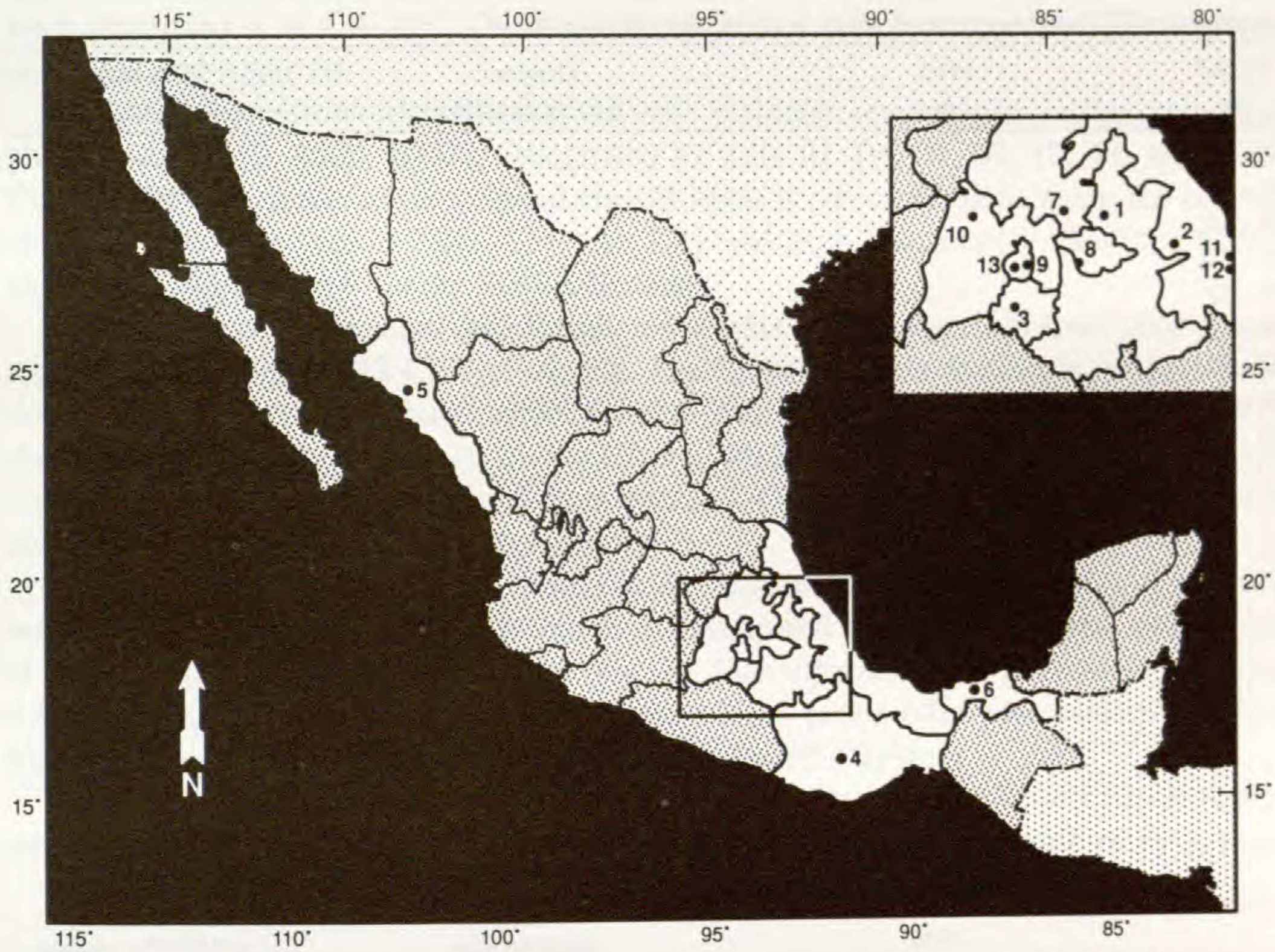

Fig. 1. Locales of *Azolla* collected in Mexico (see Table 1 for explanation of accessional code 01 - 13).

Two DNA probes were utilized in hybridizations. One was pA1HB3, a homologous random sequence of about 1.5 kb *Eco*RI ligated into pBlueScript, taken from a genomic library of *A. mexicana.* The other was pHA2, representing one pea genomic rDNA repeat unit, an 8.8 kb sequence in the *Hin*dIII site of pBR322 (Jorgensen et al., 1987).

Random amplified polymorphic DNAs (RAPDs) were obtained from all accessions by modifying a published protocol (Bassam et al., 1992). Approximately 25 ng of genomic DNA was used as template in 25 µl reactions containing 50 mM Tris HC1 pH 9.0, 5.0 mM $Mgcl_2$, 20 mM NH_4SO_3, 200 µM each dNTP, 1 µM primer, and 2.5 units *Tfl* DNA polymerase (Epicentre, Madison WI, USA). Primers (Genosys Biotech., Inc., The Woodlands TX, USA) used in this single-primer amplification technique were 10 bases in length with 50–80% G + C content.

Amplifications of DNA were performed on a MJ Research, Inc., Model 60 thermal controller (Watertown, MA). The amplification program included an initial denaturation of 1 min. at 94°C, then 35 cycles between 92°C and 30°C (1 sec. at each temperature), with a ramped heating rate of 1°C per 2 sec. Afterwards samples were given a 5 min. final elongation at 72°C, then held at 4°C overnight. Amplified DNA product was fractionated by electrophoresis in a 2% agarose gel (1% NuSieve GTG plus 1% Seakem LE; FMC Bioproducts, Rockland ME, USA) and stained with ethidium bromide for UV photography. Amplifications were always repeated at least twice to verify the consistency of results.

Allozymes. — Electrophoretic enzyme comparisons were conducted with all accessions.

Table 2. *Azolla* Lam. accessions of Mexico, original species classifications, and identification from molecular fingerprints (DNA and allozymes for 01 - 13; allozymes for the rest[a]).

EDAF No.	IRRI No.	Original Sp. Designation	Molecular Sp. Designation
01 - CH	–	–[b]	*filiculoides*
02 - JA	8028	–	CA/ME[c]
03 - MO	–	–	CA/ME
04 - OA	–	–	*filiculoides*
05 - SI	–	–	CA/ME (?) - unique
06 - TA	4139	microphylla	CA/ME
07 - HI	–	–	*filiculoides*
08 - TE	8030	–	CA/ME
09 - TL	–	–	*filiculoides*
10 - TO	–	–	*filiculoides*
11 - VI	–	–	CA/ME
12 - VII	3511	*caroliniana*	CA/ME
13 - XO	–	–	*filiculoides*
14	–	–	CA/ME
15	–	–	CA/ME
16	–	–	CA/ME
17	–	–	CA/ME
–	1510	*filiculoides*	CA/ME (?) - unique
–	2501	*mexicana*	*filiculoides*
–	3510	*caroliniana*	CA/ME
–	3519	*caroliniana*	CA/ME
–	3520	*caroliniana*	CA/ME
–	4506	*microphylla*	CA/ME (?) - unique
–	8001	–	CA/ME (?) - unique
–	8031	–	CA/ME

[a]Origins of newer accessions described in materials and methods.
[b]classified accession
[c]CA/ME = *caroliniana/mexicana*

Allozyme methodology was previously optimized for *Azolla* (Zimmerman et al., 1989a, b). Enzymes stained were glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49), isocitrate dehydrogenase (IDH; EC 1.1.1. 42), malate dehydrogenase (MDH; EC 1.1.1.37) malic enzyme (ME; EC 1.1.1.40), phosphoglucoisomerase (PGI; EC 5.3.1.9), phosphoglucomutase (PGM; EC 2.7.5.1), 6-phosphogluconate dehydrogenase (6PGD; EC 1.1.1.44), and shikimate dehydrogenase (SKDH; EC 1.1.1.25).

Results

The relative growth rates of the original 13 accessions under greenhouse conditions showed doubling times of wet-weight biomass from <4 days to >10 days. The putative FI accessions, as defined below, had six of the slowest seven growth rates and among the lowest N-accumulation rates. The most rapid RGR and N-accumulation was observed for 05-Sinaloa (2.17 g N g^{-1} dry weight y^{-1}), followed by 02-Jalapa (1.97 g N g^{-1} dry weight y^{-1}).

The results of RFLP, RAPD, and allozyme comparisons agreed in their assessments of *Azolla* species status (Table 2). The sole exception was 13-Xochimilco. The genetic fingerprints from DNA extractions at the start of this investigation unambiguously placed this accession with the FI group. However, zymograms conducted on biomass two years

later identified it as CA-ME. These results indicated that cross-contamination of that accession had occurred.

The other accessions identified as CA-ME included several with unique polymorphisms (05, 1510, 4506, 8001). Designated FI were 01, 04, 07, 09, 10, 13, and 2501. Corrections were made to the original classification of two accessions using the enzyme and DNA markers. *Azolla* 2501 – Mexico City had been inaccurately identified as ME and 1510 – Hidalgo as FI in the IRRI collection.

The pHA2 probe revealed RFLP polymorphisms within the ribosomal gene spacer regions of the original 13 CEDAF *Azolla*. The FI *Azolla* were distinguished by a band doublet at 1.2 kb in the 6-band ladder formed from hybridizations of pHA2 to *BamH*I-digested genomic DNA (Fig. 2). FI and CA-ME also differed in their patterns of middle repetitive DNA sequences obtained from genomic DNA digested with *EcoR*I or *EcoR*V and hybridized to pA1Hb3 (data not shown).

In *BamH*I-digested DNA, the pA1Hb3 probe elucidated a doublet of bands at approximately 5.5 kb and a faint band near 9kb for FI, compared to only a prominent single band at the 9-kb site for CA-ME accessions (Fig. 3). This separation was previously noted for *Azolla* accessions from other regions. The pA1Hb3 probe otherwise non-specifically hybridized to high molecular-weight DNA in the *BamH*I digest.

The RAPD fingerprints generated by a number of primers also defined *Azolla* accessions as FI or CA-ME. One example is primer CS-56 (Fig. 4); FI is identified by a major

Fig. 2. Restriction fragment length polymorphisms from a Southern blot of BamHI-digested *Azolla* genomic DNA hybridized to a pHA2 pea nuclear rDNA repeat unit. DNA size markers (kb) are located on the left margin. An asterisk marks each location of a hybrided doublet of bands. The order of accessions are: a) 01, b) 02, c) 05, d) 06, e) 07, f) 08, g) 09, h) 11, i) 12, j) 13, k) 04.

Table 3. Allozyme marker loci for designated FI and CA-ME accessions of *Azolla*. Those with unique electromorphic alleles are listed in parentheses.

Enzyme/Locus	FI Group[a]	CA/ME Group[b]
IDH	A	B
PGM-1	A[c]	B[c], (05/1510/4506)[c]
PGM-2	A (09)[d]	B (05/1510, 4506)
6PGD	A	B (06, 1510, 8001/4506)
SKDH-1	A	B (1510, 06, 8001)
SKDH-2	A (07)	B (05/06, 1510, 8001, 4506)

[a]01, 04, 07, 09, 10, 2501
[b]non-FI accessions (listed in Table 1)
[c]A = homozygous for fast allele; B = heterozygous; 5/1510/4506 = homozygous for slow allele of PGM-1
[d]A = heterozygous; 09 = homozygous for fast allele of A (differs in position from B group for PGM-2)

DNA doublet at about 915 and 875 base pairs, and CA-ME by bands at 900 and 715 bp (06-Tabasco yielded weakly amplified bands at these locations). Both taxa have a common band near 560 bp. This species dichotomy remained consistent with repeated amplifications, with template DNA from different extractions, and with *Azolla* controls from other regions.

Enzymatically, FI were identified by known allelomorphic markers (Table 3), including a slower PGM-2 locus and a faster IDH locus than those of other New World species (Zimmerman et al., 1989b). The IDH polymorphism assisted in the correction of the misidentified ME 2501 and FI 1510. Enzymes which did not show species specificity included the dimeric PGI (one locus visualized). Most *Azolla* were homozygous for the fast

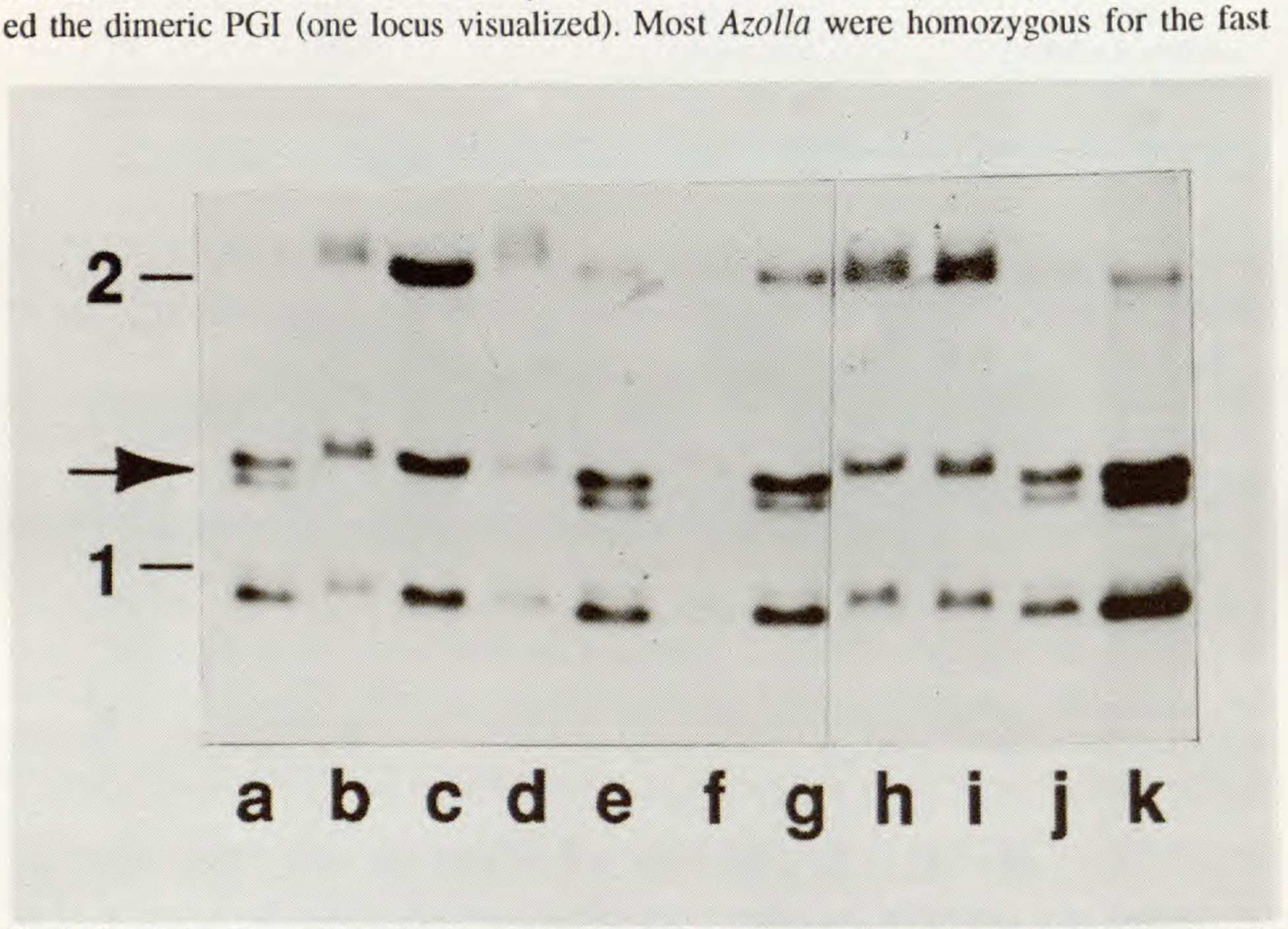

Fig. 3. Restriction fragment length polymorphisms from a hybridization of the Southern blot of Fig. 2 to the random homologous pA1Hb3 gene probe. A portion (3 of 6 bands) of the hybridization pattern is shown. An arrow indicates the location of the single or double bands which separate *Azolla* FI from CA-ME accessions. The same order of accessions are illustrated.

allele and only three were heterozygotes (1510, 4139, 13). The CA-ME *Azolla* were not as unified in their zymograms as the FI group, and individual accessions were at times polymorphic (Table 3). The status of 4139 and 4506 as MI was judged to be ambiguous from the molecular analyses. Finally, IRRI accessions 4506, 3510, and 1510 proved not be identical duplicates of CEDAF 05, 06, and 07, respectively.

Discussion

Accurate species identification of New World *Azolla* has always been an agronomic and botanical constraint. Diagnostic keys based on morphological features (Svenson, 1944; Lumpkin & Plucknett, 1982) are not reliable for most of these species. Likewise, compilation of unverified reports of *Azolla* occurrence, especially of tropical FI (Guatamala, Honduras, Colombia, Ecuador, Guyana, Trinidad), is of limited value in determining species ranges (Lumpkin & Plucknett, 1982).

Molecular fingerprinting is becoming essential in germplasm collections to check the identities (and thus the geographic origins) of *Azolla* accessions. Cluster analyses and distance phenograms from isoenzymes of the alleged taxa of *Azolla,* section Azolla, have circumscribed the CA-ME-MI group as well as the unique characters of FI and *A. rubra* (RU) (Zimmerman et al., 1989b). Direct genetic information from DNA fingerprinting has confirmed these observations (Zimmerman et al., 1991b).

This current investigation has now applied the appropriate markers to the working needs of agronomic botanists. An integrated phenetic-genetic system which identifies New World *Azolla* and the comprehensive profiling of an *Azolla* germplasm collection

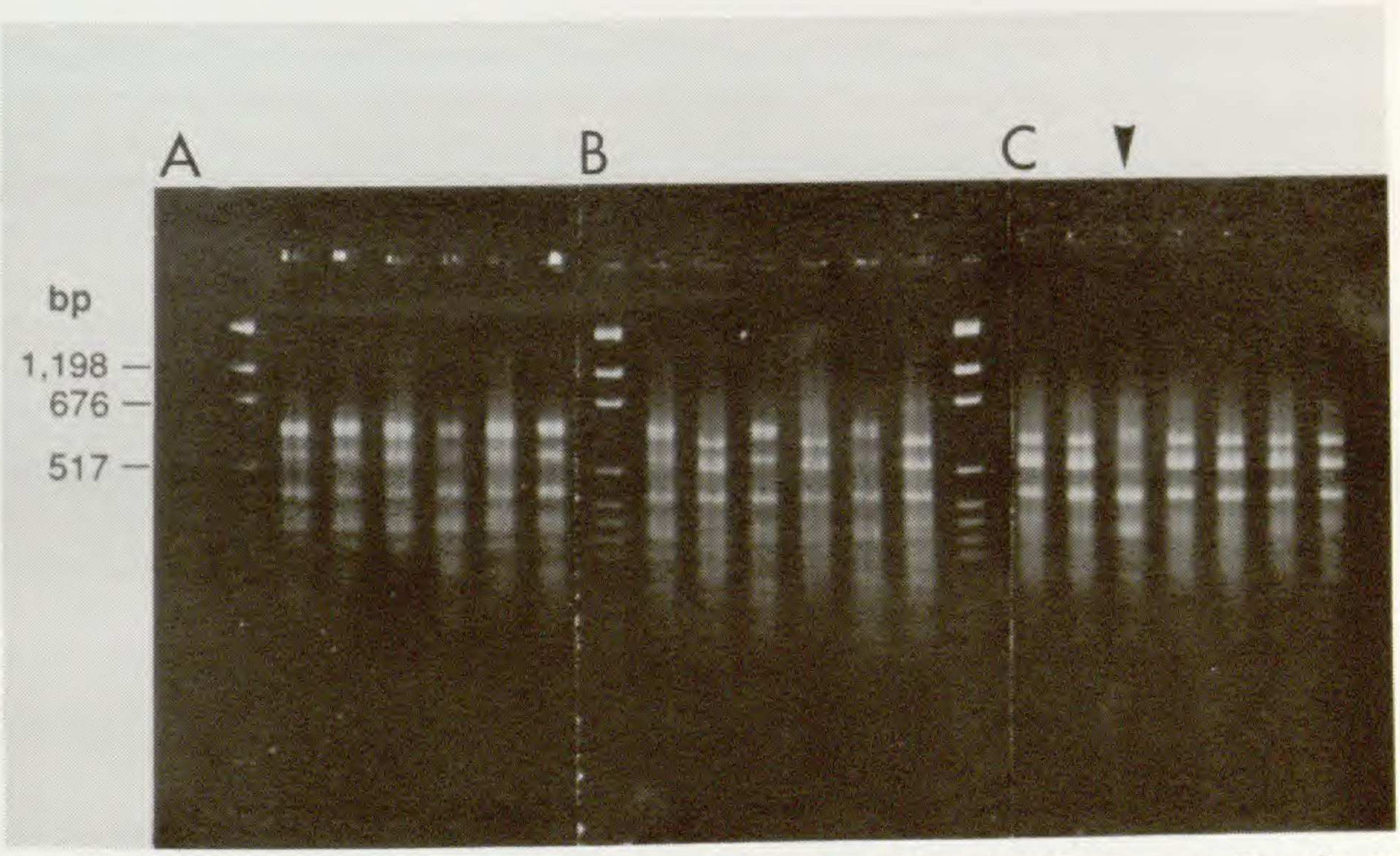

Fig. 4. Random amplified DNA polymorphisms separating *Azolla* FI from CA-ME accessions. The relevant primer was CS-56 (5' → 3' TGGTGGGTCC); reference DNA bands (base pairs) are seen in three lanes. FI accessions are located in Gel A, CA-ME in Gel C and both are compared in alternate lanes of Gel B. The arrow in Gel C indicates a slightly unusual RAPD with this primer for accession 06.

has been demonstrated for the first time. The latter objective is required to monitor cross-contamination and somaclonal mutation among the vegetative accessions.

The full distribution of *Azolla* species within Mexico is still not documented. In general, edaphic fern diversity is low except in elevated regions of the humid southeast (Palacios-Rios, 1991; Riba, 1991). Although several CEDAF *Azolla* accessions were collected in Veracruz, which is third in its species richness of ferns for all of Mexico (Palacios-Rios, 1991), one was also collected in species-poor Sinaloa. Since superior agronomic ecotypes may exist, collection of new *Azolla* of Mexico continues.

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